INFLUENCE OF CYCLOHEXIMIDE ON THE EXPRESSION OF ALKALOID METABOLISM IN PARTIALLY SYNCHRONIZED EMERGED CULTURES OF *PENICILLIUM CYCLOPIUM* WESTLING

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1. Introduction

The development of emerged cultures of *P. cyclopium* proceeds in three subsequent phases [1]:

the germination phase (0–12 hr p.i.),
the trophophase (12–48 (60) hr p.i.),
the idiophase (beginning about 48–60 hr p.i.).

The rapid increase of the protein content and mycelial dry weight in the trophophase stops about 48 hr p.i. The following idiophase is characterized by processes of morphological and chemical specialization of the culture, including the detachment and ripening of conidiospores and the formation of enzymes involved in the biosynthesis of the two benzodiazepine alkaloids cyclopenin and cyclopenol [1–3].

If certain specialization characteristics in Ascomycetes, e.g. conidiation, secondary product formation, are expressed only by emerged cultivation, it is a convenient method for a partial synchronization of these processes to conduct the growth period under submerged conditions and afterwards transfer to emerged conditions [6]. When a corresponding procedure was performed with strain SM 72a of *P. cyclopium* (cf. Materials and methods), the timing relationships and the quantitative aspects of the idiophase processes are practically unchanged as compared with those in ordinary emerged cultures [1,2]. However, there is a rapid transition from the tropho- to the idiophase and the full expression of the idiophase characteristics is accelerated [2]. The experimental bases of this culture technique, enabling the continuous registration of the alkaloid formation rates, depend on the fact that the amount of alkaloids excreted to the nutrient solution is a direct measure of the alkaloids produced in the hyphae [1] and that the increase of the alkaloid formation rate during the idiophase requires permanent RNA and protein synthesis (inhibition by ethanol, 5-fluorouracil, and cycloheximide) [2].

In this paper it is shown that the application of cycloheximide (CH) to this kind of cultures leads to a three-phasic oscillation of the alkaloid formation rate including a 'superinduction' phenomenon. Though there are a considerable number of metabolic effects following CH application, e.g. increase of soluble amino acid levels, decrease and subsequent increase of [^{3}H]phe incorporation into proteins and of β-galactosidase inducibility, evidently none of them is directly correlated to the oscillation of the alkaloid formation rate.

2. Materials and methods

The strain SM 72a of *P. cyclopium* [3] the culture conditions and the composition of the nutrient solution NLI [4], the analysis of emerged cultures (rates of alkaloid formation and incorporation of [^{3}H]phe

Abbreviations: CH: cycloheximide; cyn-col: sum of benzodiazepine alkaloids cyclopenin and cyclopenol; hr p.i.: hours after inoculation; phe: phenylalanine; TCA: trichloroacetic acid.

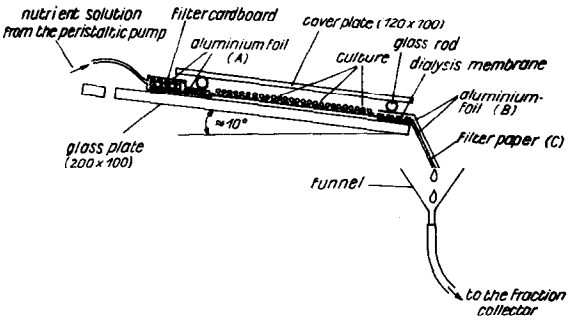

Fig.1. Schematic drawing of the equipment for cultivating *P. cyclopium* under conditions of continuous nutrient replacement.

into TCA-precipitable material) [1], and the induction and measurement of β-galactosidase [5] have been described elsewhere.

Partially synchronized emerged cultures with continuous replacement of the nutrient solution were precultivated under submerged conditions on rotatory shakers [4]. By inoculation with about 10^8 conidia/200 ml NLI globular colonies of about 1 mm diameter were obtained. 48 hr p.i. these were transferred for emerged cultivation to glass plates covered with a double sheet of filter paper and a dialysis membrane and spread out to a monolayer (fig.1). The culture was covered with a glass plate to prevent extreme evaporation, kept in a stream of sterile air (0.15 m/sec, temperature 24–25°C), and supplied via the filterpaper with nutrient solution (10 ml/hr) using NLI 0–12 hr after the beginning of the emerged cultivation and later on diluted NLI containing 15% of the original nutrient concentration. The culture broth was collected in fractions of 10 ml and analyzed for its cyn-col content [1].

3. Results and discussion

If cycloheximide (CH, 100 μg/ml) is applied to this kind of cultures, a three-phasic response is observed (fig.2B):

1. After addition of the drug the alkaloid formation rate stops immediately at the level reached before (primary inhibition).
2. Removal of CH results in an almost instantaneous 20–30 hr increase of the alkaloid formation rates surpassing those in the control cultures (superinduction).
3. There is a second halt of the alkaloid formation rate (secondary inhibition) followed by an increase essentially identical with that in the control cultures.

This kind of 40–50 hr oscillation of the alkaloid formation rates around those in the control cultures is found irrespective of the time point of CH addition and hence of the rate of alkaloid formation reached before. The 'superinduction' is most pronounced, if CH is added 40–60 hr after transfer. Evidently CH neither interferes with the alkaloid formation per se nor with the excretion of cyn-col to the nutrient solution.

Whereas the 'primary inhibition' correlating with the almost complete inhibition of the [^{3}H]phe incorporation into TCA precipitable material (fig.2A) is in agreement with earlier results in ordinary replacement cultures [2], the 'superinduction' deserves special notice. Several possibilities for an explanation must be considered:

a) The inhibition of the protein synthesis leads to a transient increase of the levels of free amino acids in the hyphae (fig.2A) with the exception of glycine, alanine, glutamic acid, and aspartic acid. On account of the rapid normalization of this effect a substrate stimulation of the alkaloid formation by the precursor amino acids phenylalanine and methionine is very unlikely. However, it cannot be excluded that the brief increase of the level of phenylalanine is connected with the subsequent superinduction phenomenon because phe evidently plays a hitherto ill-defined regulatory role for the expression of alkaloid metabolism in *P. cyclopium* [2].

b) CH was reported to stabilize polysomes in rat liver [7] and reticulocytes [8], and to cause an increased stability and storage of arginase mRNA in Aspergillus nidulans [9]. As no enhancement of β-galactosidase formation in the partially synchronized cultures of *P. cyclopium* was found after CH action (fig.2D), there is no physiological evidence for an increased polysome content or accumulation of mRNA in this case.

c) The CH-evoked protein deficiency triggers an

increased activity and (or) de novo formation of the translational apparatus after the drug has been removed. In fact, the incorporation of [^{3}H]phe into TCA precipitable material (fig.2A) as well as the inducibility of β-galactosidase (fig.2C) in the CH cultures after a period of strong inhibition increase over those in the control cultures. However, in spite of certain relationships there are also distinct differences between the dynamics of the individual processes. The good correlation between [^{3}H]phe incorporation and alkaloid formation rates 40–80 hr after transfer (fig.2A,B) is opposed to the lacking 'secondary inhibition' in the [^{3}H]phe incorporation. Furthermore, a direct connection between 'superinduction' and the overshooting of the [^{3}H]phe incorporation into proteins is doubtful because the related effect

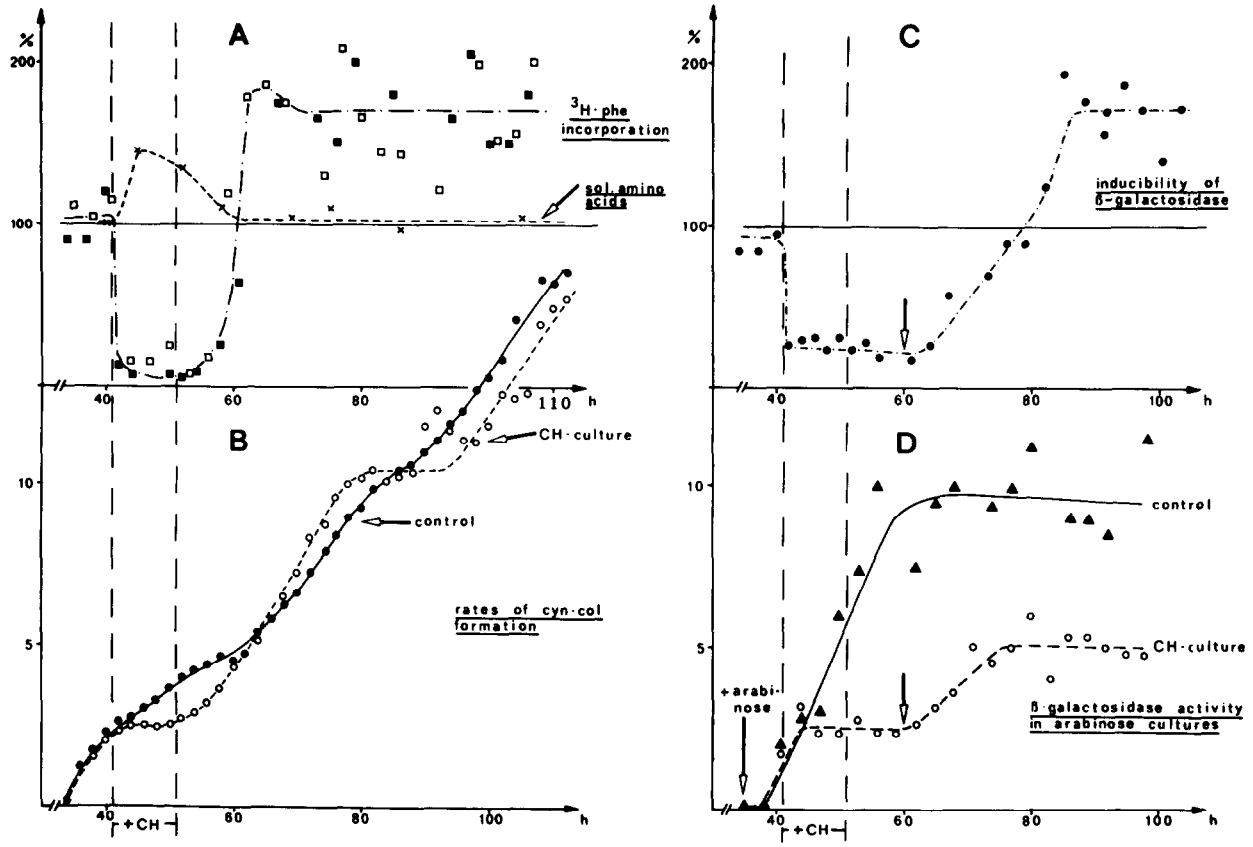

Fig. 2. Influence of cycloheximide on the levels of soluble amino acids, the [^{3}H]phe incorporation into proteins, the rates of cyn-col formation, and the induction of β-galactosidase. Cultivation as described in the experimental part. At the indicated time interval 100 μg/ml CH were added to the nutrient solution. Abscissa: hours after transfer to emerged conditions. For the determination of σ[^{3}H]phe incorporation (A) (cf. [1]). The incorporation in the control cultures (=100%) decreased during the culture period to about one half of the initial value 35 hr after transfer. The open and full squares represent two independent experiments. Free amino acids (A) were extracted from culture disks by grinding with sand in 70% alcohol. In an aliquote the amino acids were determined with ninhydrin reagent. 100% = 2.7 μmoles amino acids/cm^2 culture area calculated for leucine as the reference standard. Rates of cyn-col formation (B): 10 = 10 μg/cm^2 culture area × hr. For the measurement of β-galactosidase inducibility (C) disks of glucose cultures were kept 3 hr on a nutrient solution containing 7.5 mg/ml arabinose and afterwards analyzed for β-galactosidase as described elsewhere [5]; 100% = 30 and 6 mU/cm^2 culture area 40 hr and 100 hr after transfer, respectively. For the long-time induction of β-galactosidase (D) 35 hr after transfer the glucose nutrient solution was replaced by arabinose nutrient solution. The cyn-col formation proceeded essentially as in the glucose cultures. β-Galactosidase was analyzed as described [5]; 10 = 12 mU/cm^2 culture area.

of the model induction of β-galactosidase is observed only at a time when the dynamics of the alkaloid formation rate already pass to the 'secondary inhibition' phase (80 hr after transfer). It is interesting to notice that both, β-galactosidase inducibility in glucose cultures exposed to a 3 hr induction with arabinose (fig.2C), and β-galactosidase activity in arabinose cultures (fig.2D) increase with a 10 hr lag-phase after CH removal. At this time the [^{3}H]phe incorporation into the proteins has reached the control levels (cf. the arrows in fig.2C and D).

d) The role of metabolic unstable regulatory RNAs and proteins leading to an inactivation (and possible degradation) of the corresponding mRNAs or enzyme proteins [10–12] has been discussed in connection with related superinduction phenomena caused by numerous antibiotics, including CH, on induced enzyme synthesis in practically all kinds of organisms (cf. the summary [10]). Though the experimental evidences in some systems are very persuasive, the existence of such regulatory molecules has not been shown directly up to now. Application of this concept to the regulation of alkaloid formation in *P. cyclopium* leads to the following highly speculative explanation of the 'superinduction' phenomenon. The increase of the alkaloid formation rate in the idiophase in general is controlled by a regulatory protein interfering with the formation of the rate-limiting protein(s) of the alkaloid-synthesizing enzyme chain. CH incubation leads to a significant decrease of the cellular concentration of the regulatory protein and consequently after restoration of protein synthesis to an abnormally high rate of the de novo formation of the supposed rate-limiting protein(s).

It is evident that there is no direct correlation between the oscillation of the alkaloid formation rates after CH application and the other CH effects described. The further analysis of these phenomena necessitates an in vitro determination of the hitherto unknown rate-limiting protein(s) of alkaloid biosynthesis. The latter problem is tightly connected with investigations about the possible role of enzyme degradation for the expression of the alkaloid metabolism in general and after CH application in particular.

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